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Title page

Title:

Electrospray ionization mass spectrometry for the hydrolysis complexes of cisplatin:
implications for the hydrolysis process of platinum complexes

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Abstract

Non-enzyme dependent hydrolysis of the drug cisplatin is important for its mode of action and toxicity. However, up until today, the hydrolysis process of cisplatin is still not completely understood. In the present study, the hydrolysis of cisplatin in an aqueous solution was systematically investigated using electrospray ionization mass spectrometry coupled to liquid chromatography. A variety of previously unreported hydrolysis complexes corresponding to mono-, di-, and trimeric species were detected and identified. The characteristics of the Pt-containing complexes were investigated using collision induced dissociation (CID). The hydrolysis complexes demonstrate distinctive and correlative CID characteristics, which provides tools for an informative identification. The most frequently observed dissociation mechanism was sequential loss of NH_3 , H_2O , and HCl . Loss of the Pt atom was observed as the final step during the CID process. The formation mechanisms of the observed complexes were explored and experimentally examined. The strongly bound dimeric species, which existed in solution, are assumed to be formed from the clustering of the parent compound and its mono- or dihydrated complexes. The role of the electrospray process in the formation of some of the observed ions was also evaluated and the ESI related cold clusters were identified. The previously reported hydrolysis equilibria were tested and subsequently refined via a hydrolysis study resulting in a renewed mechanistic equilibrium system of cisplatin as proposed from our results.

Keywords

Cisplatin, Platinum complexes, ESI-MS, Hydrolysis, Identification

Main text

Introduction

Platinum complexes are a major class of chemotherapeutic agents against cancer^[1, 2], with cisplatin (*cis*-diamminedichloroplatinum (II)), carboplatin (*cis*-diammine 1,1-cyclobutane dicarboxylato platinum (II)), oxaliplatin (1*R*, 2*R* -diaminocyclohexane) oxalatoplatinum (II)), and nedaplatin (*cis*-diammine-glycolatoplatinum (II)) being first-line representatives used for the treatment of a wide variety of cancers such as ovarian and testicular carcinomas^[3-7]. Even though the platinum complexes have been widely used for cancer therapies since the first member cisplatin was discovered in the 1960s^[8], the mechanisms of action and toxicity for these coordination complexes are still not completely understood^[5, 9, 10]. It is generally accepted that the mode of action of platinum complexes consists of binding of the hydrolyzed complexes to the DNA. This requires activation through non-enzyme dependent hydrolysis. Through this binding to the DNA and reaction with other biologically important molecules^[7, 8, 11, 12], they exert an anticancer activity but also cause undesirable side effects such as cytotoxicity and nephrotoxicity^[8, 13, 14]. Therefore, understanding the hydrolysis process of platinum complexes is of considerable significance to elucidate the mechanisms behind the anticancer and toxic effects and also to pave the way for the development of new coordination complexes in the fight against cancer.

The hydrolysis of cisplatin has previously been explored through various types of conventional techniques such as HPLC-UV, atomic absorption spectrometry (AAS), flame atomic absorption spectroscopy (FAAS), X-ray, electrochemical detection, inductively coupled plasma-mass spectrometry (ICP-MS), HPLC-ICP-MS, and nuclear magnetic resonance (NMR)^[15-17]. A schematic overview of the different hydrolysis products of cisplatin in water is summarized in Figure 1^[10, 18, 19]. Some of the aforementioned techniques cannot differentiate parent drug and its hydrolysis species since they only measure total platinum. A typical example of this is presented by Hann et al. who reported on a detailed hydrolysis speciation of cisplatin via HPLC-ICP-MS^[20]. In this study, large amounts of unknown Pt-species were found in the hydrolysis solution along with cisplatin, and its monohydrated complex (MHC) and dihydrated complex (DHC). Furthermore, only NMR spectrometry can provide definitive structural information on cisplatin and its hydrolysis products^[6, 21].

However, in order to obtain NMR spectra, at least high microgram to milligram amounts of material are required^[6, 10]. For platinum complexes which are almost insoluble in most organic solvents (e.g. methanol and acetonitrile) and are not stable in aqueous solution^[22], this is very challenging. In addition to this, relatively pure samples of target compounds are often required for these techniques, which makes the reliable detection of these unstable complexes even more challenging. Due to these limitations, up until today, the hydrolysis scheme of cisplatin is still not completely clarified^[20]. Therefore, it is worthwhile to experimentally re-examine the reported hydrolysis process using a technique which is able to provide structural information such as electrospray ionization mass spectrometry (ESI-MS).

ESI-MS is a powerful and versatile technique for structural identification of metal-containing aqueous solutions. A variety of platinum and palladium complexes containing various ligands have been previously investigated using ESI-MS^[23-25]. Nevertheless, only limited mass spectral data regarding the hydrolysis of cisplatin is available in the literature^[10, 18]. Ehrsson and coworkers^[18] were the first to study the characteristics of cisplatin in water using ESI-MS. They identified the sodium adduct of cisplatin, and proton and acetonitrile-adducts of the mono- and dihydrated products. In this study, the spectra were recorded in a relatively narrow mass range m/z 10 - 500, and only limited full scan spectra and fragmentation information was used for the identification. M. Cui et al. reported a detailed ESI-MS spectrum as well as a fragmentation pathway of cisplatin and its hydrated complexes^[10]. A hydroxo-bridged dimer of the dihydrated species was identified in their study. However, three abundant species located at the range of m/z 500 - 580 in the full scan ESI mass spectrum were left unidentified.

It should be noted that the oligomeric complexes observed via ESI-MS are not always a true reflection of the composition of the species in solution. Sometimes cold clusters are produced in the electrospray process. This is most likely to occur when an electrosprayed solution contains neutral as well as charged species^[5, 26]. Therefore, care should be taken when an oligomer is found and additional efforts should be devoted to elucidate whether such an oligomer is produced in the solution or is actually a cold cluster of the ESI-MS process.

The above mentioned difficulties are overcome when ESI-MS is hyphenated with the resolving power of an LC system. Then, it is possible to elucidate what ESI-MS species are formed, and where (i.e. in solution or gas phase or both) and how they are formed. The in-depth understanding of the aqueous chemistry of cisplatin contributes to the general knowledge of the hydrolysis process of platinum (II) and potentially also palladium(II) complexes for which cisplatin could be regarded as a model compound.

The goal of present study was to 1) provide a comprehensive and thorough understanding of the hydrolysis process of cisplatin and identify all hydrolysis products; 2) generate kinetic information on the degradation pathway and the overall equilibrium of cisplatin in aqueous solution.

Experimental

Reagents

Cisplatin was purchased from the EDQM Council of Europe. ULC-MS grade water and isopropanol were obtained from Biosolve (Valkenswaard, The Netherlands). Formic acid and ammonium formate were supplied from Sigma-Aldrich (Belgium).

Hydrolysis of cisplatin

The stock solution (± 1 mM, pH 5.85) was freshly prepared by dissolving approximately 3.0 mg cisplatin in 10.0 mL of ULC-MS water with a resistivity of 18.2 - 30 Mohm·cm. The bottle was closed and heated to 37 °C in an oven. Aliquots were removed from this solution at pre-defined time points according to the purpose of study. For the ESI-MS infusion study, aliquots were taken from the solution at 0, 4, 24, and 72 h. These solutions were diluted 5-fold with ULC-MS water prior to infusion. For UPLC-ESI-MS/MS experiments, the aliquots were taken at 0, 0.5, 1, 2, 3, 4, 5, 7, 24, 27, 30, 48, and 55 h. These samples were immediately stored at -80 °C until analysis. Afterwards the samples were allowed to thaw and were diluted 10-fold with ULC-MS water and subsequently 4-fold with acetone prior to injection onto the UPLC system.

Liquid chromatography

Chromatographic separations were performed on a Synchronis HILIC column (50 x 2.1 mm, 1.7 μ m, Fisher Scientific, Aalst, Belgium) installed on an Acquity UPLC H-class system (Waters, Milford, MA). The mobile phase (MP) consisted of MP A (10/85/5 isopropanol/water/100 mM ammonium formate buffer, pH 2.75) and MP B (95/5 isopropanol/100 mM ammonium formate buffer, pH 2.75). Components were eluted using a linear gradient at a flow rate of 0.4 mL/min which consisted of the following sequence: 0-3.0 min, from 95% MP B to 60% MP B; 3.0-3.5 min, from 60% MP B to 95% MP B; 3.5-5.5 min, 95% MP B. The column temperature was maintained at 45 °C, and the injection volume was 2 μ L.

Mass spectrometry

All MS experiments were performed using a Waters Xevo TQS tandem mass spectrometer (Waters, Milford, MA) operated in positive electrospray ionization (ESI⁺) mode. The desolvation gas (nitrogen) flow rate was set to 1000 L/h at a temperature of 500 °C, and collision gas (argon) flow was maintained at 0.15 mL/min. The source temperature was maintained at 125°C. For ESI-MS experiments, all data were collected and analyzed using Masslynx 4.1 software using MS scan (full scan) and MS/MS modes. The MS scan spectra were acquired in the mass range of 100 - 1000 *m/z* for 2.0 mins. The electrospray capillary voltage and cone voltage were optimized at 3.0 kV and 20 V, respectively, to maintain satisfactory sensitivity and circumvent in-source fragmentation. The collision energy (CE) used for the collision-induced dissociation (CID) was tested on a low, medium, and high setting (4-70 eV) for each species. The aqueous solutions were introduced to the source at a flow rate of 20 μ L/min. For LC-ESI-MS/MS experiments, the hydrolysis complexes were detected in multiple reaction monitoring (MRM) mode with a dwell time of 0.020 s per transition. Details of the optimized MRM transitions and parameters for each species are presented in Table 1.

Results and discussion

ESI-MS scan and CID experiments

Hydrolysis complexes containing different numbers of platinum and chloride atoms are fairly straightforwardly distinguished via ESI-MS due to the naturally occurring stable isotopes of

platinum and chloride. The isotopic distribution for platinum starts at 190.0 Da (0.014 %) and continues up to 198.0 Da (7.16 %) with the most abundant mass at 195.0 Da (33.83 %). Chloride gives two abundant isotopes at 35.0 Da (75.78 %) and 37.0 Da (24.22 %). The theoretical isotope distributions for molecules containing Pt_nCl_n elements are summarized in supplementary Table S1. The identification of the observed parent and product ions containing Pt and Cl elements is achieved through the comparison of the experimentally observed isotopic clusters with the calculated expected isotope cluster patterns by using the windows-based IsoPatrn program^[27]. In this study, the mass containing the most abundant isotope for each element in the molecule is used for the ESI-MS scan and CID experiments, while the most abundant mass for each of the complexes is used for the LC-ESI-MS/MS experiments.

The positive ion mass spectra obtained from a 5-fold dilution of an 1 mM aqueous solution of cisplatin kept at 37°C for 0 and 72 h, respectively, are given in Figure 2a and 2b. No significant ions were found below m/z 200, above m/z 900 or between m/z 350 - 500 and m/z 650 - 800. Therefore, the full scan mass spectra in the mass range of m/z 200 - 900 (Figure 2a and Figure 2b) and expanded spectra in the mass range of m/z 220 - 340 (Figure 2c), m/z 510 - 640 (Figure 2d), and m/z 820 - 880 (Figure 2e) were presented. Electrospray of the fresh solution (0 h) of cisplatin gives most abundant $[\text{M}+\text{NH}_4]^+$ (m/z 317.0), $[\text{M}+\text{Na}]^+$ (m/z 322.0) ions, and abundant $[\text{M}+\text{K}]^+$ (m/z 338.0), $[2\text{M}+\text{NH}_4]^+$ (m/z 616.0), $[2\text{M}+\text{Na}]^+$ (m/z 621.0) ions (Figure 2a), while no protonation of parent complex is observed. A variety of supplemental ions were observed in the mass spectra of the hydrolyzed solution (72 h) of cisplatin (Figure 2b). These ions correspond to newly formed mono-, di-, and trimeric species. The signal of the base peak at m/z 283.0 in this spectrum is beyond the MS maximum response (1.34×10^8). An additional scan for this specie, was acquired from a 20-fold dilution of the hydrolysis solution, giving a response of 1.17×10^8 . In order to obtain the actual relative abundances of other species observed in Figure 2b, the theoretical response of the base peak in Figure 2b (5-fold dilution) is calculated by multiplying the non-saturated response for the 20-fold dilution base peak (1.17×10^8) by the dilution ratio (20 / 5). The thus obtained base peak intensity for the spectrum can then be used as the basis for the relative abundance calculations. This assumes a linear MS response. Next, a series of CID experiments at varying collision energies were undertaken to elucidate the structures. The MS/MS spectra were

documented in Figures S1 - 20. Based on the information of the charge states, molecular masses, isotope distribution patterns, and fragmentation pathways obtained from ESI-MS and CID spectra, the structures of the observed ions were identified. An illustration of these structures can be found in Figure 1 and Figures S1 – 8, 11, 15, 16. Based on the number of Pt atoms contained in the species, the structures are divided into three subgroups, i.e. monomeric, dimeric, and trimeric cations.

Monomeric cations

Upon dissolving cisplatin in water, it is expected that MHC (i.e. the monoaqua complex and monohydroxo complex) and DHC (i.e. the diaqua complex, monohydroxylated mono aqua complex, and dihydroxylated complex) are formed. The presence of MHC in our solution was supported by the ions at m/z 281 - 287 and 303 - 307, which corresponded to the monoaqua complex and Na-adduct of the monohydroxo complex, respectively. Moreover, the ions (m/z 263 - 267) of the monohydroxylated mono aqua complex and the dihydroxylated complex were detected, while a doubly charged $[M]^{2+}$ ion (m/z 132) of the diaqua complex was not detected.

It should be noted that the monoaqua complex ($[M]^+$, m/z 282.0) suffers from in-source fragmentation when the cone voltage exceeds 40 V, giving fragmentation ions $[M-H_2O]^+$ (m/z 264.0) and $[M-H_2O-HCl]^+$ (m/z 228.0). Similar to MHC, DHC is also quite sensitive to the cone voltage, giving fragmentation ions $[M-H_2O]^+$ (m/z 246.0) and $[M-2H_2O]^+$ (m/z 228.0). Furthermore, this ion specie suffers from the interferences of in-source fragmentation ions $[M-H_2O]^+$ (m/z 263 - 267) of MHC. Therefore, careful tuning of the cone voltage is crucial for detecting DHC. In the present study, the cone voltage could not exceed 25 V to avoid in-source fragmentation of DHC and to obtain the authentic isotopic pattern. This is likely the reason why the dihydrated complex was not observed in a previous series of hydrolysis investigations of $Pd(en)Br_2$ ^[28], $Pd(en)Cl_2$ ^[5], and $Pt(en)Cl_2$ ^[7] (en: ethylenediamine) in which a high cone voltage was used.

Two molecular ions at m/z 291 - 295 and m/z 273 - 277 were identified as $Pt^+(NH_3)_2(N_2)Cl$ and $Pt^+(NH_3)_2(N_2)(OH)$. These species result from the clustering of ion-source fragmentation ions $[M-H_2O]^+$ of MHC and DHC, respectively, with desolvation gas N_2 . The $Pt^+(NH_3)_2(N_2)Cl$ ion

has been confirmed in a recent hydrolysis investigation of cisplatin using high-resolution mass spectrometry^[6], and a similar phenomenon was also observed during the CID process of $\text{Pt}(\text{en})\text{Cl}_2$ ^[5].

Dimeric cations

Dimer 4 ($[\text{M}]^+$, m/z 563.0): The ions in the mass spectrum which clustered at m/z 561 - 569, having a relative abundance of 6.8 %, was interpreted as the $\text{Pt}(\text{NH}_3)_2\text{Cl}_2 \cdot \text{Pt}^+(\text{NH}_3)_2\text{Cl}$ dimeric complex. The overall single positive charge is the result of the loss of a chloride ion in the complex. It is interesting to see that only moderately abundant $\text{Pt}(\text{NH}_3)_2\text{Cl}_2 \cdot \text{Pt}^+(\text{NH}_3)_2\text{Cl}$ was found in our ESI-MS study, while the similar dimeric species $\text{M}(\text{en})\text{X}_2 \cdot \text{M}^+(\text{en})\text{X}$ (M: Pd, Pt, X: Cl, Br) are observed as the base peak ions in the previous ESI-MS hydrolysis investigations^[5, 7, 28]. This is most probably caused by the different pH in the hydrolysis solution since the species observed are quite pH dependent.

When the ion $[\text{M}]^+$ at m/z 563.0 is subjected to CID, the fragment ions at m/z 546.0 and m/z 529.0 were produced by sequential loss of one, respectively two NH_3 groups (Figure S16). The fragmentation ions at m/z 510 and 508 correspond to the neutral loss of HCl from m/z 546.0. Further fragmentation results in the loss of Pt and chloride atoms containing groups, yielding product ions clustering at m/z 263.0, m/z 246.0, m/z 227.0, m/z 210.0, which were already discussed in the above monomeric cations section (for reference, see the supplementary materials). The dimeric complex did not break apart into its monomer subunit $\text{Pt}^+(\text{NH}_3)_2\text{Cl}$ during initial CID experiments, contrary to what we would expect for a non-covalently bound complex. Instead, the sequential loss of NH_3 and HCl occurred, indicating a strong interaction (probably covalent interaction) between the subunits $\text{Pt}(\text{NH}_3)_2\text{Cl}_2$ and $\text{Pt}^+(\text{NH}_3)_2\text{Cl}$.

The observed $\text{Pt}(\text{NH}_3)_2\text{Cl}_2 \cdot \text{Pt}^+(\text{NH}_3)_2\text{Cl}$ ion is likely to be formed from the reaction of the neutral $\text{Pt}(\text{NH}_3)_2\text{Cl}_2$ with its monoaqua complex $\text{Pt}(\text{NH}_3)_2\text{Cl}(\text{H}_2\text{O})^+$. The ESI-MS properties of cisplatin and its hydrolysis products can also be investigated by looking at its analogs (e.g. $\text{Pd}(\text{en})\text{Br}_2$, $\text{Pd}(\text{en})\text{Cl}_2$, $\text{Pt}(\text{en})\text{Cl}_2$), which are expected to hydrolyze in an analogous way as cisplatin. The density functional theory (DFT) calculations used in the previous hydrolysis study of $\text{Pt}(\text{en})\text{Cl}_2$ demonstrated that the clustering reaction of parent drug and its

monoaqua complex is favored in the gas phase and only slightly endergonic in solution, suggesting that the formation of this dimeric species is possible both in solution and during the ESI-MS process^[7]. An aggregation formation pathway is also proposed by the same authors^[7]. That is, a dimeric structure clustered from two intact parent molecules is first formed, then a chloride is lost to yield the observed ion. This pathway is theoretically possible in solution, while it is predicted to be highly endergonic in the gas phase. Therefore, the formation mechanism for this dimer is still unclear when only the ESI-MS and DFT calculations were employed, requiring further investigations using LC-MS/MS (see further on).

Dimer 3 ($[M]^+$, m/z 545.0): The observation of a complex with the most abundant ion at m/z 545.0 and a relative abundance of 8.6 % is consistent with the $\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)_2\text{Cl}$ ion (Figure 2b). CID of the $\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)_2\text{Cl}$ ion at m/z 545.0 (Figure S15) leads to three fragment ions at m/z 528.0, 510.0 and 493.0 by sequential loss of NH_3 , H_2O and NH_3 . Further fragmentation of m/z 493.0 yields the ions at m/z 457.0 and 455.0, 421.0 and 419.0, as well as 404 and 402 via successive loss of two HCl and one NH_3 molecules. Finally, the ion at m/z 404 and 402 produces the similar cluster of ions at m/z 263.0, m/z 246.0, m/z 227.0, m/z 210.0 as dimer 4 due to the loss of Pt and Cl containing species.

A. Yoshikawa et al.^[7] explained the formation of the $\text{Pt}(\text{en})(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{en})\text{Cl}$ ion via the clustering of the neutral $[\text{M}-\text{HCl}]$ (M: $\text{Pt}(\text{en})\text{Cl}_2$) ion with the $[\text{M}-\text{Cl}+\text{H}_2\text{O}]^+$ ion, followed by proton transfer from the bridging H_2O group to the deprotonated amino group. Instead, we believe that the $\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)_2\text{Cl}$ ion is formed from clustering of the monohydroxo complex ($\text{Pt}(\text{NH}_3)_2\text{Cl}(\text{OH})$) and the monoaqua complex ($\text{Pt}(\text{NH}_3)_2\text{Cl}(\text{H}_2\text{O})^+$) via a hydroxo-bridging interaction by the loss of H_2O .

Dimer 2 ($[M]^+$, m/z 527.0): The ions starting at m/z 525 - 534 with a relative abundance of 1.9 % were attributed to an overlay of the ions of dimer 2 ($\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)_2(\text{OH})$, m/z 527.0) and an in-source fragment ion $[\text{M}-\text{NH}_3]^+$ ($\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)\text{Cl}$, m/z 528.0) of dimer 3. The theoretical isotope distribution with corresponding intensities for the $\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)_2(\text{OH})$ ion starts from m/z 525 (35.5 %) to 533 (5.36 %) with the most abundant mass being m/z 527 (100 %). The pattern starting from m/z 526 (32.4 %) to

534 (11.3 %) with the most abundant mass at m/z 528 (100 %) is thought to originate from the in-source fragment ion $\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)\text{Cl}$. The relative peak intensities of the two overlapping complexes (m/z 525 - 534) was influenced by the cone voltage (Figure S9). This provided us with a means to distinguish between these two ions. At a cone voltage of 20 V, when only weak in-source fragmentation would occur, the most abundant ion of the cluster ions (m/z 525 - 534) was at m/z 527.0 with an isotope pattern of 37.1: 70.6: 100 at m/z 525 - 527. This corresponds to the $\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)_2(\text{OH})$ ion containing two Pt and one Cl atoms. At a cone voltage of 45 V, the most abundant ion became m/z 528.0 with a clustering pattern of 40.1: 71.4: 100: 89.3: 81.0: 49.9: 36.7: 13.6: 10.6 at m/z 526 - 534. This pattern is in excellent agreement with the theoretical values for an ion containing two Pt and two Cl elements. The increase in the signals of the ion at m/z 528.0 and decrease in the response of the dimer 3 at high cone voltages indicated that the $\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)\text{Cl}$ ion probably results from in-source fragmentation of dimer 3.

Further support for this hypothesis comes from a series of CID experiments at m/z 525 and 527 at a cone voltage of 20 V (Figures S10 and S12), and at m/z 525, 528, and 534 at a cone voltage of 45 V (Figures S11, S13, and S14). The ions at m/z 525 (cone voltage: 20 and 45 V) and 527 (cone voltage: 20 V) demonstrated identical fragment patterns via the loss of H_2O , NH_3 and HCl (Figures S10 and S11). This confirmed the presence of two Pt and one Cl elements in the proposed structure: $\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)_2(\text{OH})$. When the ions at m/z 528 and 534 (cone voltage: 45 V) undergo CID (Figures S13 and S14), a similar fragmentation pattern was observed as discussed above for dimer 3. This offers the confidence for ascribing the $\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)\text{Cl}$ ion to the in-source fragment ion of dimer 3.

The formation of dimer 2 ($\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)_2(\text{OH})$) can also be explained by assuming the hydroxo-bridging of the monohydroxo complex ($\text{Pt}(\text{NH}_3)_2\text{Cl}(\text{OH})$) and the monohydroxylated mono aqua complex ($\text{Pt}(\text{NH}_3)_2(\text{OH})(\text{H}_2\text{O})^+$) via the loss of H_2O . This is supported by the presence of these two hydrolyzed species.

Dimer 5 ($[\text{M}+\text{Na}]^+$, m/z 603.0): The cation observed at m/z 601 - 609 with a relative abundance of 1.8 % is assumed to be sodium adduct of $\text{Pt}(\text{NH}_3)_2\text{Cl}(\text{OH}) \cdot \text{Pt}(\text{NH}_3)_2\text{Cl}_2$ complex resulting from clustering of the monohydroxo complex ($\text{Pt}(\text{NH}_3)_2\text{Cl}(\text{OH})$) and intact cisplatin

via non-covalent bonds. The weak nature of this interaction is corroborated by the CID where the $\text{Pt}(\text{NH}_3)_2\text{Cl}(\text{OH})\text{Na}^+$ ion was easily formed with 100 % relative abundance, through the loss of neutral cisplatin (Figure S18).

Trimeric cations

Clustering of neutral cisplatin with dimer 2, 3, and 4 (m/z 826.0, 844.0, and 862.0) resulted in three extremely low abundant (0.15 - 0.2 %) ion clusters at m/z 823 - 833, 841 - 852 and 859 - 871. These are assumed to be the $\text{Pt}(\text{NH}_3)_2\text{Cl}_2 \cdot \text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)_2(\text{OH})$, $\text{Pt}(\text{NH}_3)_2\text{Cl}_2 \cdot \text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)_2\text{Cl}$, and $\text{Pt}(\text{NH}_3)_2\text{Cl}_2 \cdot \text{Pt}(\text{NH}_3)_2\text{Cl}_2 \cdot \text{Pt}^+(\text{NH}_3)_2\text{Cl}$ ions. They are likely to be formed via non covalent interactions between cisplatin and dimer 2, 3, and 4, respectively. Although CID experiments for these clusters were not performed due to the lack of sensitivity, the non-covalent bound properties of these complexes can be learned from the reported trimetallic cations (i.e. $[\text{3M-HCl-Cl+H}_2\text{O}]^+$ and $[\text{3M-Cl}]^+$) of $\text{Pt}(\text{en})\text{Cl}_2$ ^[7].

As demonstrated in the above analysis, various monomeric cations of cisplatin, MHC, and DHC were detected. This is in line with previous hydrolysis studies of cisplatin^[10, 18]. Nevertheless, we discovered a number of previously unreported monomeric cations such as the Na-adduct of the monohydroxo complex and $\text{Pt}^+(\text{NH}_3)_2(\text{N}_2)(\text{OH})$. Furthermore, the previously reported hydroxo-bridged dimer^[10] (dimer 1) of the dihydrated complex, as well as the previously unreported $[\text{2M+NH}_4]^+$, $[\text{2M+Na}]^+$ (both of cisplatin) (Figures S19 and S20) and $[\text{2M+Na}]^+$ (of the monohydroxo complex) ions (Figure S17) were also observed in our study. Besides these previously unreported monomeric cations, more interestingly, a variety of unreported di- and trimeric cations were detected in our hydrolysis study.

LC-ESI-MS/MS experiments

As discussed above, the formation mechanisms of the observed (dimeric) complexes cannot be fully elucidated via ESI-MS infusion studies alone. Thus, LC-ESI-MS/MS (See supporting documents for key comments on the LC-MS/MS) was used to further confirm our findings and elucidate how and when the species are formed (i.e. in solution or in the gas phase or both). The typical chromatogram obtained from a 40-fold dilution of a sample of cisplatin which was incubated at 37°C for 55 h is shown in Figure 3. The peak areas as a function of time for cisplatin and its hydrolyzed species are shown in Figure 4.

The peak eluting at 1.78 min in Figure 3 (a) is intact cisplatin and the small peak at 1.37 min is assumed to be transplatin since it shows similar MS/MS behaviour as cisplatin. In our study, cisplatin undergoes a hydrolytic degradation to 28.8% of its initial value after 24 h. This finding is consistent with earlier literature reports^[10, 29, 30]. It was also reported that the cis-trans isomerization of cisplatin occurs in aqueous solutions and is increased at elevated temperatures^[31]. As shown in Figure 4, transplatin is formed gradually when dissolving cisplatin in water, reaching a plateau after 24 h. We calculated that approximately 7.6 % of initial cisplatin is converted to transplatin after 24 h. This was based on the assumption that the MS/MS response is similar between cisplatin and transplatin.

The presence of MHC is observed from the peak eluting at 1.94 min in Figure 3 (c). The peak area of MHC increases over 24 h along with the degradation of cisplatin. As seen from Figure 4, it is the most abundant hydrolysis product. As illustrated in Figure S21 (a), DHC presented itself as a broad and tailing peak at 2.9 - 4.4 min, Adjusting the elution conditions (e.g. pH of the mobile phase, and gradient settings) did not significantly improve the peak shape. The dihydrated dimer gave rise to a minor peak at 3.75 min (Figure S21 (b)). In an undiluted hydrolysis solution nevertheless, it showed an abundant intensity in the corresponding ESI-MS spectrum. This indicates that the dihydrated dimer probably decomposes readily in the applied (acidic) elution conditions. This is conformed in earlier work by Cui^[10] and Berners-Price^[32].

Dimer 4, shown in Figure 3 (b), eluting simultaneously with cisplatin, was gradually formed over 24 h. Afterwards, its concentration remained stable for the remainder of the incubation time interval. As discussed above, dimer 4 can be formed through the reaction of neutral cisplatin with its monoaqua complex or through an aggregation pathway. The aggregation pathway, however, is not supported by our experimental results since an increasing formation leveling out from 7 h onwards was observed for dimer 4. If aggregation would occur in solution, an initial increase followed by a decrease is expected for dimer 4 due to the decreasing cisplatin concentrations. A similar, decreasing concentration-time pattern comparable to cisplatin is also expected for dimer 4 when the aggregation happens in the gas phase. The formation of dimer 4 in the gas phase via a clustering reaction is

equally not supported by our results since cisplatin and its monoaqua complex are well separated and would therefore not exist simultaneously in the gas phase. Our analysis indicated that dimer 4 is most likely to be formed in solution via a clustering reaction between cisplatin with its monoaqua complex.

For dimer 3, two peaks were found. As seen from Figure 3 (d), one peak eluted at 1.93 min (peak 1) and another at 2.60 min (peak 2). Peak 1 always demonstrated an identical retention with MHC, even under various elution conditions. Furthermore, the peak area ratios of MHC to peak 1 are stable throughout the whole incubation time. Therefore, we assume that peak 1 is formed in the gas phase via the clustering of the monohydroxo complex and the monoaqua complex. Peak 2, however, is regarded as the authentic form of dimer 3 and is formed in solution since it is chromatographically separated from MHC. Its formation time profile is presented in Figure 4. Our results demonstrate that dimer 3 can be formed both in the gas phase and in solution via clustering of the monohydroxo and the monoaqua complexes.

The specific transition $528.0 > 264.0$ of the observed $\text{Pt}(\text{NH}_3)(\text{OH}) \cdot \text{Pt}^+(\text{NH}_3)_2\text{Cl}$ ion shows two peaks in Figure 3 (e), labeled as peak 3 and peak 4. Their retention times are consistent with peak 1 and peak 2 of dimer 3. The peak area ratios of peak 1 to peak 3 and peak 2 to peak 4 are identical and behave similar over the whole 55 h incubation time interval, indicating that this species is an in-source fragment of dimer 3, confirming our ESI-MS observations regarding this ion.

Similarly, the peak shown in Figure 3 (f) is presumed to be dimer 2. Although it elutes at the same retention time as MHC, the peak area ratios of MHC to dimer 2 are different up until the moment when the hydrolysis equilibrium is reached. Therefore, the dimer 2 is more likely to be formed in the solution via the reaction of the monohydroxo and monohydroxylated mono aqua complexes. This can also be confirmed from its extremely low abundance since the monohydroxylated mono aqua complex is found in very low contents.

The weakly bound dimer 5 showed a poorly shaped peak centered at 1.80 min, with a baseline ranging from 1.60 min to 2.1 min (Figure S21 (c)). The formation pathway for dimer

5 is unclear since it is almost undetectable after the 40-fold dilution of the hydrolysis solution.

The LC-ESI-MS/MS data confirmed that there were not only cisplatin and its mono- and dihydrated complexes in the hydrolysis solution, but also their clustering dimers (dimer 1 - 4). It is worth noting that in our study, three unreported dimers (dimer 2 - 4), adding up to 17.3% relative abundance, were detected in the hydrolysis solution. These dimers were in equilibrium with monomeric species. In a previous hydrolysis study^[10], the mono- and dihydrated species, and dihydrated dimer species (dimer 1) were found using the same initial concentration of cisplatin. The three unidentified species from this study, located at m/z 500 - 580, are consistent with dimer 2 - 4 which were observed in our study. Based on these newly found dimers in hydrolyzed solution, a renewed equilibrium system for cisplatin is presented in Figure 1, previously unreported.

Conclusions

Our ESI-MS study identified a number of previously unreported Pt-containing complexes. These correspond to mono-, di-, and trimeric complexes of cisplatin, of which the most abundant complex is the monohydrate. The structures of the complexes were elucidated by combining full scan MS data, data on expected/observed isotope patterns, MS/MS and LC-ESI-MS/MS experiments. The strongly bound $\text{Pt}(\text{NH}_3)_2\text{Cl}_2 \cdot \text{Pt}^+(\text{NH}_3)_2\text{Cl}$ and $\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)_2(\text{OH})$ dimeric species are believed to be the only ones formed in solution, whereas the $\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)_2\text{Cl}$ ion can be formed both in solution and during the ESI process. The observed $\text{Pt}^+(\text{NH}_3)_2(\text{N}_2)\text{Cl}$, $\text{Pt}^+(\text{NH}_3)_2(\text{N}_2)(\text{OH})$, and $\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)\text{Cl}$ ions were qualified as cold clusters. The formation of the $\text{Pt}(\text{NH}_3)_2\text{Cl}_2 \cdot \text{Pt}^+(\text{NH}_3)_2\text{Cl}$, $\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)_2\text{Cl}$ and $\text{Pt}(\text{NH}_3)_2(\text{OH})\text{Cl} \cdot \text{Pt}^+(\text{NH}_3)_2(\text{OH})$ bimetallic cations is hypothesized to originate via clustering reactions of cisplatin and its mono- and dihydrated species. At the same time, three weakly bound trimetallic ions were observed, arising from ion-molecule interactions between three bimetallic cations and cisplatin. Our study of the hydrolysis kinetics of cisplatin has revealed a rich and complicated aqueous chemistry and based on these results a new equilibrium system of cisplatin in H_2O is proposed.

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Table

Table 1. Detection parameters for the different species under study.

Species	Cone voltage (V)	Collision voltage (kV)	MRM transitions
Dimer 1	25,0	12,0	245.76 > 228.24
DHC	20,0	14,0	264.04 > 227.99
MHC	25,0	8,0	283.01 > 265.00
Cisplatin	25,0	14,0	318.11 > 265.00
Dimer 2	20,0	18,0	527.01 > 473.96
Pt(NH ₃)(OH) · Pt ⁺ (NH ₃) ₂ Cl ion	45,0	25,0	528.01 > 264.01
Dimer 3	20,0	10,0	544.97 > 527.95
Dimer 4	20,0	12,0	564.99 > 547.97

Figure captions

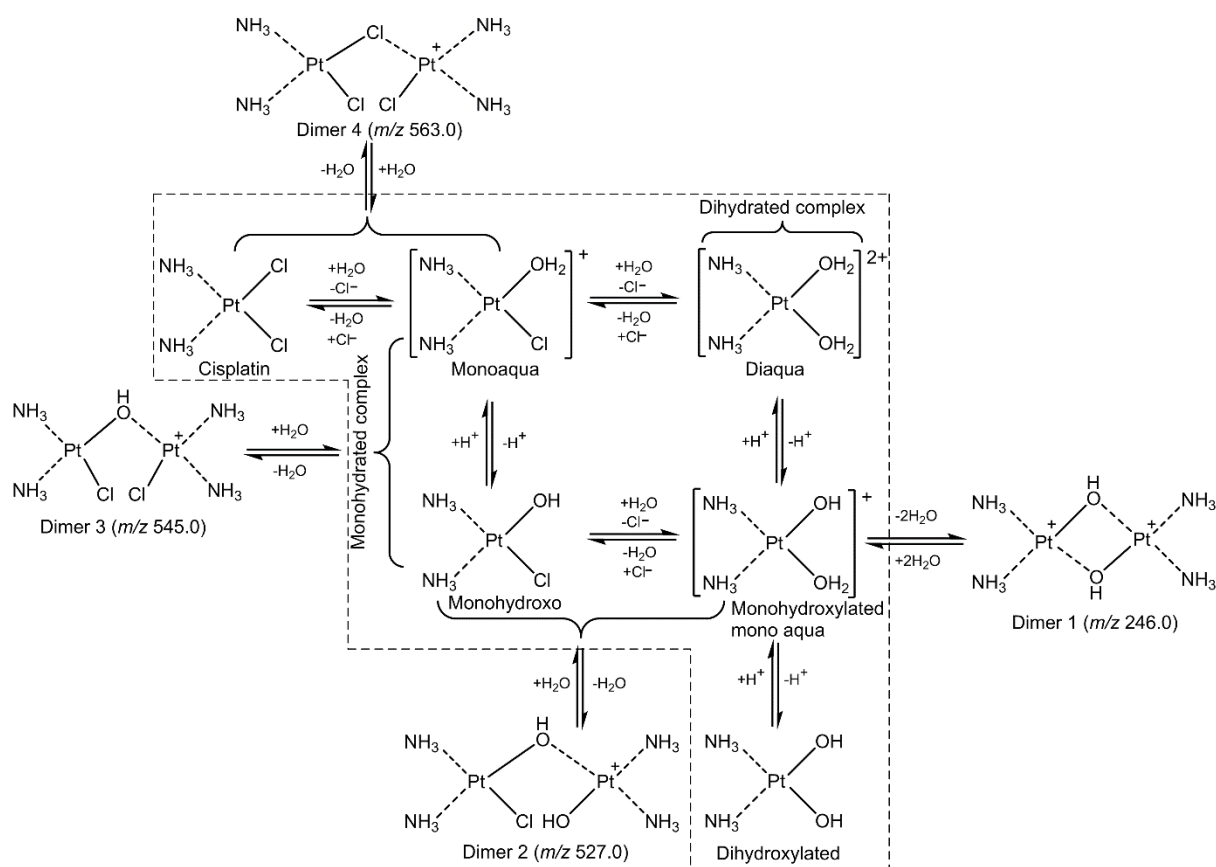


Figure 1 The previously reported (marked by the dashed line) and updated hydrolysis equilibria of cisplatin in water.

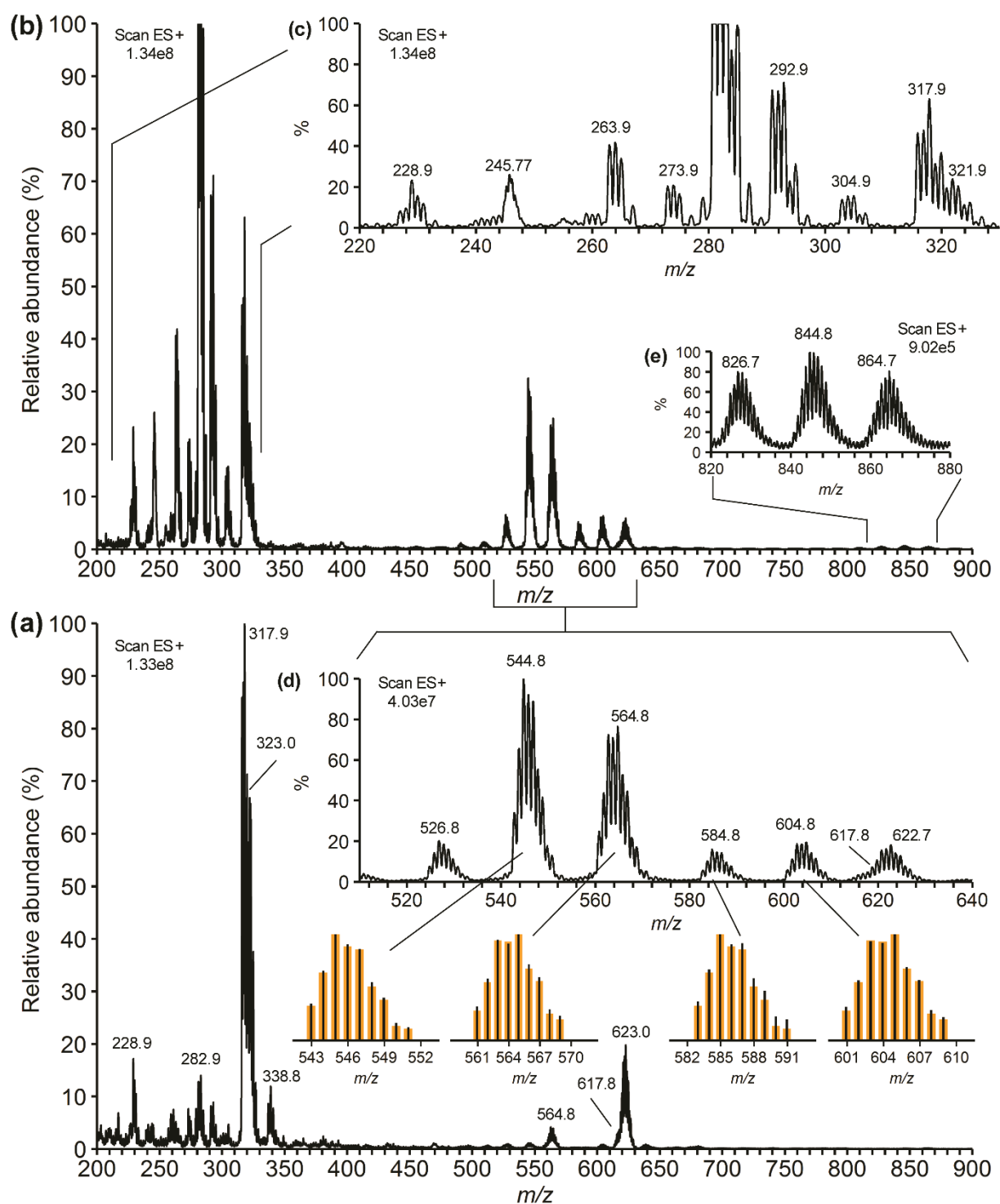


Figure 2 ESI-MS spectra of a 5-fold dilution of an 1 mM aqueous solution of cisplatin incubated at 37°C for 0 h (a) and 72 h (b). (c) Expanded spectrum in the range of m/z 220 - 340. (d) Expanded spectrum in the range of m/z 510 - 640. (e) Expanded spectrum in the range of m/z 820 - 880. Inserts: experimental (lines) and theoretically calculated (bars) isotopic distributions of assigned peaks.

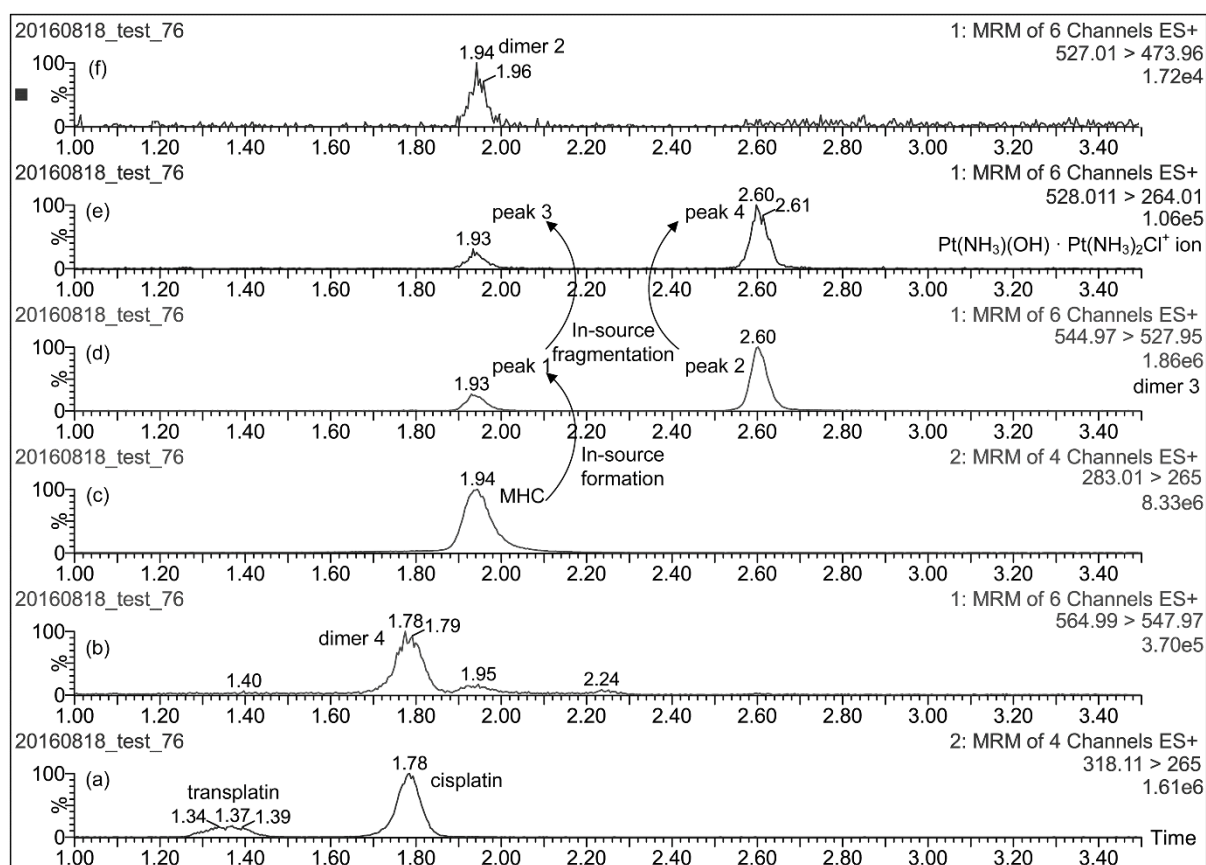


Figure 3 Representative LC-ESI-MS/MS chromatograms of cisplatin and its hydrolysis complexes obtained from a 40-fold dilution of a solution of 1 mM (initial) cisplatin, incubated for 55 h at 37°C. (a) LC-ESI-MS chromatograms of cisplatin (1.78 min) and transplatin (1.37 min). (b) LC-ESI-MS chromatogram of dimer 4 (1.78 min). (c) LC-ESI-MS chromatogram of MHC (1.94 min). (d) LC-ESI-MS chromatogram of dimer 3: peak 1 (1.93 min, in source formation via clustering of the monohydroxo complex and the monoaqua complex) and peak 2 (2.60 min, formation in solution via clustering of the monohydroxo and the monoaqua complexes). (e) LC-ESI-MS chromatogram of the $\text{Pt}(\text{NH}_3)(\text{OH}) \cdot \text{Pt}^+(\text{NH}_3)_2\text{Cl}^-$ ion, an in-source fragment ion of dimer 3. (f) LC-ESI-MS chromatogram of dimer 2 (1.94 min).

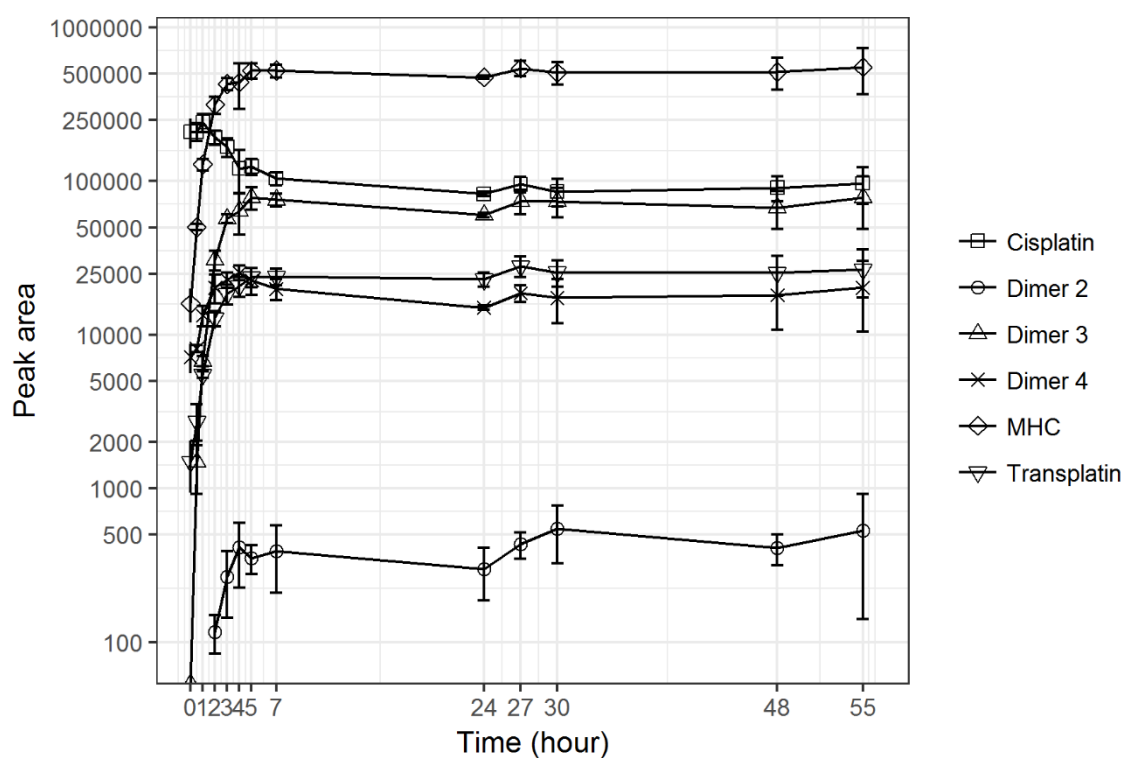


Figure 4 Time resolved patterns of mean peak areas and their 95% confidence intervals of cisplatin and its hydrolysis species obtained from a 40-fold dilution of a solution of 1 mM (initial) cisplatin, incubated for 55 h at 37°C.